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(54) Title: PROTECTIVE PEPTIDE ANTIGEN CORRESPONDING TO PLASMODIUM FALCIPARUM CIRCUMSPOROZOITE PROTEIN

(57) Abstract

A protective peptide antigen corresponding to Plasmodium falciparum circumsporozoite. A DNA encoding the peptide is also disclosed. The peptide tandemly repeats at least twenty three times and comprises eptopes of Plasmodium falciparum CS protein.

	Pro	Asn	Ala	Asn	
G[15]	CCA	AAT	GCA	AAC	
	C	T	A	C	
	A	C	A	C	
	C	T	A	T	
	T	T	A	C	
	C	T	A	T	
	T	T	A	T	
	T	T	C	T	
	A	T	A	T	
	T	T	A	C	
	C	T	A	T	
	T	T	A	T	
	T	T	C	T	
	A	T	A	T	
	A	T	A	C	
	A	C	A	C	
	C	T	A	T	
	T	T	C	T	
	A	T	A	T	
	A	T	A	C	
	A	T	A	C	
	A	T	A	C	
	C	T	A	T	
	CCT	AAT	AAA	AAC	
	AAT	CAA	GCC	CCC	C[18]

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PROTECTIVE PEPTIDE ANTIGEN CORRESPONDING TO PLASMODIUM FALCIPARUM CIRCUMSPOROZOITE PROTEIN

The Government has rights in this invention based upon research support in the form of Grant No. 5RO1-AI-17429-03 from the Department of Health and Human Services and Grant No. AID-DPE-0453-C-00-2002-00 from the Department of State, Agency for International Development.

Background of the Invention

The present invention relates to an antigen suitable for providing protective immunity against malaria e.g. by incorporation into a vaccine. A formidable health problem in large areas of the world, malaria affects more than 150 million people in any given year. Of the four plasmodial species which cause malaria in humans, *Plasmodium falciparum* is responsible for most of the severe infections and the highest rate of mortality. Combating malarial infestations caused by *P.falciparum* has become more difficult due to the spread of drug-resistant organisms in many areas. The occurrence of severe epidemic outbreaks of this disease lends particular urgency to recent efforts to develop a malaria vaccine.

Under normal conditions, a malarial infection is initiated by the introduction of sporozoites into the bloodstream of the host through the bite of infected mosquitoes. Hence, inactivation of these sporozoites by the immune system of the host could completely block development of the infection. Several recent findings

point to the f asibility of developing an antisporozoite vaccin . Sporozoit s are highly immunog nic and are capable of eliciting a prot ctive immune response in several host species, including man: se e.g. Cochrane, A.H. et al. Malaria, Vol. 3, J.D. Kreier, Ed. (Academic Press, New York 1980), pp. 163-202. The immunogenicity of sporozoites resides largely, if not exclusively, in a single antigen, the circumsporozoite (CS) protein (described in detail by F. Zavala, A.H. Cochrane, E.H. Nardin, R.S. Nussenzweig, V. Nussenzweig, J. Exp. Med. 157: 1947 (1983), which covers the entire parasite surface, as reported by M. Aikawa, N. Yoshida, R.S. Nussenzweig and V. Nussenzweig in Journal of Immunology, 126: 2494 (1981). Finally, the immunogenicity of the CS protein is restricted almost entirely to a single epitope which is identically or quasi-identically repeated several times in tandem: G.N. Godson, et al. Nature 305: 29 (1983); V. Enea et al., accepted for publication Proc. Nat'l. Acad. Sci. (1984).

Identification of the amino acid sequence of CS epitopes for all plasmodial species that infect humans is a prerequisite for the development of a human synthetic sporozoite vaccine.

Several monoclonal antibodies have been raised against the CS protein of _Plasmodium falciparum_ sporozoites. These antibodies inactivate the parasites. Methods for obtaining such antibodies are well known in the art and have been disclosed by Nardin E. et al. in J. Exp. Med. 156: 20 (1982), and in U.S. Patent Application Serial No. 234,096 of Nussenzweig et al, filed February 12, 1981 the disclosure of which is incorporated herein by reference. (The disclosure of this application also incorporates by reference the entire disclosure of assignee's copending U.S. Patent Application Serial No. 574,553 filed January 27, 1984 of Nussenzweig, et al. entitled Protective Peptide Antigen).

These monoclonal antibodie against CS protein bind to a repeated epitope, which is common to different isolates of parasites obtained from different geographical areas. Such antibodies can be used to screen clones expressing peptides having or incorporating the amino acid sequence of the CS repetitive epitopes.

Antibodies against the sporozoite antigens have been shown to provide protective immunity against the plasmodium species from which they were derived, in rodents, monkeys and in human volunteers. The sporozoite protective antigen is herein termed CS protein, or circumsporozoite protein, or sporozoite CS protein, these terms being deemed equivalent and used interchangeably. Assignee's copending U.S. Patent Application of Nussenzweig, Serial No. 234,096 filed February 12, 1981 discloses a vaccine based upon purified CS protein. Assignee's copending application Serial No. 574,553 discloses a peptide comprising an epitope of a sporozoite CS protein.

The results disclosed herein are based in part on techniques and concepts in the field of immunology. For convenience, certain terms commonly used in the art are defined herein. The term "immunochemical reaction" is used to denote the specific interaction which occurs between an antigen and its corresponding antibody, regardless of the method of measurement. Such a reaction is characterized by a non-covalent binding of one or more antibody molecules to one or more antigen molecules. The immunochemical reaction may be detected by a large variety of immunoassays known in the art. The terms "immunogenic" or "antigenic" are used here to describe the capacity of a given substance to stimulate the production of antibodies specifically immunoreactive to a substance when that substance is administered to a suitable test animal under conditions known to elicit antibody production. The term "protective antigen" refers to the ability of a given

immunogen to confer resistance in a suitable host, against a given pathogen. The term "epitope", refers to a specific antibody binding sit on an antigen. Macromolecular antigens such as proteins typically have several epitopes with distinctive antibody binding specificities. Different epitopes of the same antigen are distinguishable with the aid of monoclonal antibodies which, due to their high degree of specificity, are directed against a single epitope. Two different monoclonal antibodies directed against different epitopes on the same antigen may bind the antigen without interfering with the other, unless the epitopes are so close together that the binding of one sterically inhibits the binding of the other. The term "immunodominant region" denotes an area of the antigen molecule which is mainly responsible for its antigenicity.

Summary of the Invention

The present invention involves the discovery that the protective CS sporozoite antigens of *P. falciparum* possess an immunodominant region composed of four amino acids (proline-asparagine-alanine-asparagine) that are tandemly repeated at least 23 times. The repeat comprises 8 variants at the nucleotide level. Both asparagine codons, three of the four proline codons and two of the four alanine codons are employed. This repeated sequence has been shown to contain the epitope of the CS protein of *Plasmodium falciparum*. Analogs of the repeated peptide have been chemically synthesized and have been found to be immunochemically reactive with polyclonal antibody preparations against *Plasmodium falciparum*. In addition, monoclonal antibodies against CS proteins, which neutralize the infectivity of sporozoites *in vitro*, also react with the synthetic peptide. Vaccines made with three and six tandem repeats of the four amino acid sequence (12-MER and 24-MER peptides) confer immunity to *P. falciparum* sporozoites. Thus, these synthetic peptides exhibit the protective antigenic features of the *P.falciparum* CS protein.

D tailed Description of the Invention

In the following d scription, th materials employed were commercially available, unless otherwise specified. Enzymes used in th cloning procedures were obtained from commercial sources. Restriction endonuclease reactions were carried out according to the manufacturer's instructions. Unless otherwise specified, the reaction conditions for other enzyme reactions were standard conditions used in the art, as described, for example, in Methods in Enzymology, (Vol. 68, R.Wu, Ed.) Academic Press, (1980). Unless otherwise specified, the abbreviations herein are standard abbreviations acceptable for publication in scientific journals normally used by those skilled in the art to publish their results, such as those cited herein.

Monoclonal antibodies to P.falciparum sporozoites were isolated from mouse ascites injected with hybridomas produced by fusing the spleen cells of P.falciparum sporozoite-hyperimmunized mice with NS1 myeloma cells as described in Nardin, E.H., et al J. Exp. Med. 156:20-30 (1982). The monoclonal antibody used to identify the clone expressing the protective peptide antigen of the present invention was prepared according to the procedures of Nardin, et al., supra, and designated "2A10."

In general outline, the experiments and conclusions following from the results thereof are set forth. The synthetic protein of the present invention was defined and initially secured by cloning a cDNA made from mRNA obtained from infected mosquitoes. A cDNA library was constructed from poly $(A)^+$ RNA derived from Plasmodium falciparum infected mosquitoes. Double-stranded cDNA was inserted at the PstI site of plasmid pBR322 using the dC -dG tailing method to generate recombinant plasmids that could express the inserts as a fusion protein with the beta-lactamase ncoded by the vector. Bacterial host cells (LE 392 derived from E. coli K-12) wer transformed

and the resulting t tracyclin r sistant DNA molecules were screened for the expression of CS antigen using an in situ filter immunoassay.

Approximately 10,000 colonies wer screened by an in situ radioimmunoassay with the monoclonal anti-sporozoite antibody (2A10) and a strongly positive clone, designated p277-19 was identified.

Extracts of the host bacterium LE392, harboring the plasmid p277-19 were then tested in a two-site immuno-radiometric assay by using monoclonal antibody 2A10 immobilized in plastic wells, and the same [^{125}I]-labelled antibody in the fluid phase: F. Zavala et al, Nature 229: 737 (1982).

The recombinant protein expressed by clone p277-19 is able to bind simultaneously both the immobilized and the radiolabelled antibody. This indicates that the recombinant protein, as the authentic CS protein, contains at least two epitopes which are recognized by the anti-CS monoclonal antibody 2A10.

The nucleotide sequence of the p277-19 insert is illustrated in Fig. 1. In the protein encoded by this sequence, the amino acid sequence proline, asparagine, alanine and asparagine is repeated 23 times in tandem with no variations. This repetitive pattern of four amino acids is the shortest of the known CS protein repeats. The repeats of P. knowlesi and P. cynomolgi (Gombak strain), two simian malaria parasites, are twelve and eleven amino acids long, respectively, Godson, et al. Nature 305: 29 (1983); V. Enea et al. supra (1984).

Although neither the DNA nor the protein sequences of these repeated peptides are related to one another, certain similarities are apparent from an analysis of their amino acid composition. Thus, alanine and asparagine are present in the repeats of all known CS proteins; proline is present in P. knowlesi and P. falciparum; and glutamic acid and glycin are present in P. knowl si and P. cynomolgi (gombak).

The pres nt findings indicate that the immunodominant epitope of the CS protein of P. falciparum consists of a sequence of amino acids which does not appear to require further modification to be antigenic.

EXAMPLE I.

Preparation of Plasmodium falciparum RNA

RNA was prepared from the thoraces of *Anopheles balabacensis* mosquitoes infected with *Plasmodium falciparum* of the Thai K-1 stain. The collected thoracic tissue (from 1837 mosquitoes) was homogenized in 10 volumes of 4 M guanidine isothiocyanate (pH 5.0) and 0.1M 2-mercaptoethanol (Liu et al, Proc. Nat'l Acad. Sci. (USA) 76:4503 1979; Ellis et al, Nature 302:536 (1983). The homogenate was centrifuged at 9,000 rpm for three minutes in a Sorval (RC2-6) centrifuge. The supernatant was then layered over 0.2 volumes of 5.7M cesium chloride and 0.1 EDTA (pH 6.5) and centrifuged in an SW-41 rotor at 28K for 16 to 20 hours at 20°C. The RNA pellet was resuspended in 7.5M guanidine hydrochloride in 25 mM sodium citrate (pH 7.0) with 5 mM beta-mercaptoethanol. The RNA was precipitated by adding one fortieth volume, 1 M acetic acid and one half volume of 95% ethanol at -20°C for two to three hours (Chirgwin, et al. Biochem. 18:5294 1979). This was followed by a second precipitation in 0.3M sodium acetate (pH5) and 2.5 volumes of 95% ethanol, overnight at -20°C. Following centrifugation the RNA pellet was resuspended in water and stored at -70°C.

EXAMPLE II.

Purification of the Poly(A)$^+$ RNA

Poly (A)$^+$ RNA was prepared according to the method of Aviv and Leder Proc. Nat'l Acad. Sci. (USA) 69:1408 (1972). The RNA was heated at 68°C for 10 minutes, then chilled on ice for 5 minutes. After warming the RNA sample to room temperature, binding buffer was added to a final concentation of 0.5M sodium chlorid , 0.01M Tris-HCl

(pH 7.4) and 0.01M EDTA (pH 7.0). The RNA was cycled 3-5 times through an oligo(dT) cellulose (Collaborative Res arch, Inc., Waltham, Mass.) column with a bed volume of 0.2 - 0.4 ml. The poly(A^+) RNA was lut d from the column with sterile water at room temperature. The RNA was recovered by precipitation with ethanol, and stored in water at -70°C.

Prior to the cDNA synthesis, 1.5 micrograms of the poly$(A)^+$ RNA was mixed with 75 nanograms of rabbit globin mRNA (Bethesda Research Laboratories, (BRL), Bethesda, Md.) extracted first with phenol and chloroform (1:1 v/v) and then with chloroform. The RNA was precipitated in 0.3 M sodium acetate and 2 and $^1/2$ volumes of 95% ethanol. The pellet was resuspended in six microliters of water and then stored at -70°C.

EXAMPLE III.

Construction of the cDNA Library from Poly (A)+ RNA

The first and second strands of the cDNA were synthesized by a modification of the procedure of Okayama and Berg, Molecular and Cellular Biology 2:161 (1982). Approximately 1.5 micrograms of *P. falciparum* poly$(A)^+$ RNA mixed with 75 nanograms of rabbit globin mRNA (Bethesda Research Laboratories), were incubated in a 30 microliter reaction volume containing 50 mm Tris-HCl (pH 8.3), 50 mm KCl, 8 mm $MgCl_2$, 2 mm dithiothreitol, 30 micrograms/ml oligo-$dT_{(12-18)}$ cellulose (Collaborative Research), 100 micrograms/ml Actinomycin-D (Sigma Chemical Co., St. Louis, Mo.), 100 micrograms/ml BSA (bovine serum albumin), 0.25 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP, 50 x 10^{-6} Ci alpha-[^{32}P] dATP (specific activity 3,000 curies per millimole) and 120 units of reverse transcriptase (BRL) at 42°C for 2 hours.

The reaction was stopped by extraction with phenol and chloroform (1:1 v/v), then with an equal volume of chloroform and precipitated two times with 2 M ammonium

ac tate and ethanol. The pellet was wash d with 80% thanol, dri d by d ssication under vacuum, and resuspended in 46.9 microliters of water.

The s cond strand was synthesized in a 65 microliter reaction volume containing 20 mM of Tris-HCl (pH 7.4), 4 mM of magnesium chloride, 10 mM $(NH_4)_2SO_4$, 0.1 mM of KCl, 50 micrograms/ml BSA, 0.3 mM nicotinamide adenine dinucleotide (NAD) oxidized (Sigma), 0.1 mM each of the deoxynucleotide triphosphates (dATP, dCTP, dTTP, dGTP[1/]), one unit DNA Polymerase I (Boerhinger Mannheim Biochemical, Indianapolis, Indiana), 1.5 units RNAase H (BRL), 1 unit E.Coli ligase (P.L. Biochemical). The reactants were first incubated at 15°C for one hour, then at room temperature for one hour. Again, the reaction was stopped by extraction, first with phenol/ chloroform (1:1) and then with an equal volume of chloroform. The mixture was precipitated once with 2M ammonium acetate and ethanol and the pellet washed with 80% ethanol, dried and resuspended in 4.5 microliters of water.

The second strand synthesis reaction was completed with T_4 DNA polymerase (BRL). The double stranded cDNA was incubated in 50 mM Tris-HCl (pH 8.0), 6 mM magnesium chloride, 25 mM KCl, 0.1 mM each of dATP, dCTP, dGTP and dTTP and 3.5 units of T_4 DNA Polymerase at 37°C for 30 minutes. The reaction was stopped by the addition of 25 mM EDTA. The reaction mixture was extracted with phenol and chloroform (1:1 v/v), and then with an equal volume of chloroform, followed by three washes with ether. The double-stranded cDNA was then precipitated with 0.5 M NaCl and 10% PEG (polyethylene glycol, average molecular weight 8,000) at 4°C overnight. The double-stranded cDNA was tailed with deoxycytidine residues according to Roychoudhury, et al. Nucl. Acids Res., 3, 101 (1976); Land et al. Nucl. Acids Res. 9:2251 (1981). Double-stranded cDNA (30 to 60 nanograms) were incubated in a 25 microliter

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1/ This and all dNTP's were from P.L. Biochem., Milwaukee, Wisc.

r action volum with 0.1 M potassium cacodylat (pH 7.0), 0.5 mM dCTP, 0.1 mM DTT and 2 mM $CoCl_2$ at 37°C for five minutes. Ten units of terminal d oxynucleotidyl transferase (Enzo Biochemical, Inc.) were added and the mixtur was incubated at room temperature for 1 minute. The reaction was stopped by the addition of EDTA to 10 mM. Two micrograms of yeast tRNA were added and the mixture was extracted twice with phenol/chloroform (1:1) and once with chloroform, and then precipitated with 2M ammonium acetate and ethanol.

The deoxy(C)-tailed double-stranded cDNA was resuspended in 60 microliters annealing buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 1 mM EDTA). The concentration was estimated to be 0.6 to 3 micrograms/microliter. The tailed cDNA was annealed (using the method of Land, et al. supra 1981) to PstI-cut and deoxy(G)-tailed pBR 322 (New England Nuclear) at varying ratios to determine the optimal ratio of insert to vector.

All of the pilot annealings were performed at a concentration of 250 nanograms pBR322/ml in a 200 microliter reaction volume by mixing 50 ng of pBR322 with 20, 8.0, 4.2 and 3.3 microliters of the tailed double-stranded cDNA. The 20 and 4.2 microliter pilot reactions yielded the maximum number of colonies and therefore were scaled up to make larger preparations for transformation.

EXAMPLE IV

Transformation of Host Cells

E. coli LE 392 cells were used as the bacterial host (P. Leder, et al. Science 196:175 (1977)). This is a variant of the E. coli K-12 strain. However, transformation may also be carried out in other host cells such as, DH1 available from the E. Coli Genetic Stock Center, Yale Univ. (CGSC No. 6040).

The host cells w r transformed by the recombinant plasmids using a modification of the procedure of Hanahan et al. J. Mol. Biol., 166: 557-580 (1983). 2.5 nanograms of the hybrid plasmid were added to 210 microliters of competent LE 392 cells. The mixture was incubated on ice for 30 minutes, heat shocked at 42°C for 90 seconds and placed on ice for 1 to 2 minutes. 800 microliters of SOC (2% Bactotryptone (Difco Detroit, Mich.), 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) were added and the reaction was incubated at 37°C shaking at 225 rpm for one hour. The cells were centrifuged at 2,000 rpm for 10 minutes and resuspended in 0.4 milliliters SOB without magnesium (2% Bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl) and spread on two Hanahan plates (1% Bactotryptone, 0.95% yeast extract, 10 mM NaCl, 1.5% Bacto agar) with 12.5 micrograms/ml of tetracycline (Sigma). The transformation efficiency was approximately 10^5 transformants per microgram of annealed DNA.

EXAMPLE V

Screening for the cDNA Library

The cDNA library was screened by a modification of the *in situ* radioimmunoassay as described by Helfman, et al. Proc. Nat'l Acad. Sci., (U.S.A.) 80: 31-35 (1983), as described by Enea et al, supra.

The bacteria were transferred onto 82 mM nitrocellulose filters (Millipore HATF Millipore, Bedford, Mass.). Replica filters were made and regrown on the tetracycline plates described above, at 37°C.

The bacterial colonies were lysed by placing the open petri dishes over 1 ml of chloroform for 15 minutes. The filters were then placed in individual petri dishes or pooled in trays containing 50 mm Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM magnesium chloride, 0.1 mM PMSF (phenylmethylsulfonylfluoride, BRL) 3% BSA, 40 micrograms/ml lysosome, 1 microgram/ml, DNAase I and gently

agitat d at room temperature for 1 to 2 hours. The filters were rinsed in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl for 1 to 2 hours and th n incubat d for 15 to 30 minut s in 50 mM Tris-HCl(pH 7.5), 150 mM NaCl, and 3% BSA. The filters were then incubated in a 50 ml volume with 50 X 10^6 cpm [^{125}I]-labelled monoclonal antibody 2A10 in 150 mM NaCl, 3% BSA with gentle rocking at room temperature overnight. The filters were washed extensively with 150 mM NaCl, 0.1% NP40 (Sigma), and 50 mM Tris-HCl (pH 7.5) air dried and mounted for autoradiography.

After screening approximately 10,000 colonies, one was found to react with the monoclonal antibody 2A10.

The clone was purified by streaking on LB plates (10% Bactotryptone, 50% yeast extract, 170 mM NaCl, 1.5% Bacto agar) containing 12.5 micrograms/ml of tetracycline.

A single colony was picked and tested by both the <u>in situ</u> radioimmunoassay procedure described above and the two-site radioimmunoassay (Ellis et al. Nature, Vol. 302: 536-538) (1983). In this procedure antibody 2A10 was adsorbed to the wells of a microtiter plate. Crude lysates of the bacterial clones to be tested were added to the wells and incubated for sufficient time to allow the immunoreactive protein present in the lysate to bind to the adsorbed monoclonal antibody. The wells were then washed to remove any contaminating proteins and radio-labelled monoclonal antibody 2A10 was added. The labelled antibody attached to the antigenic protein that is already bound to the surface of the microtiter well by the first monoclonal antibody. Extracts of LE 392 harboring the plasmid scored positive in this assay.

<u>EXAMPLE VI</u>

<u>Nucleotide Sequencing of Clone p277-19</u>

Plasmid DNA was prepared from LE392 (p277-19) using a modification of the method of Birnbaum et al.

Nucleic Acid R search 7: 1515 -1523 (1979). Bri fly, bacterial c lls w re grown in LB m dium (containing 10g Bactotryptone, 5g Bacto yeast extract, 10g NaCl [adjusted to pH 7.5 with NaOH] per liter) either to saturation or to an optical density of OD-600 nm of approximately 0.4 in which case chloramphenicol was added to 0.17 mg/ml. The cultures were incubated by centrifugation and resuspended in approximately 20 volumes of 50mM glucose. 25mM Tris-HCl (pH 8), 10mM EDTA and 2 volumes of 0.2N NaOH and 1% SDS were added. After incubating the suspension on ice for 10 minutes, 1.5 volume of 5M potassium acetate (pH 4.8) was added. Following a 10 minute incubation on ice, the sample was centrifuged at 8,000 rpm for 60 minutes in a fixed angle Sorval rotor and the supernatant was collected and combined with 0.6 volumes of isopropanol. The precipitate was then resuspended in 10 mM Tris, 10 mM EDTA (pH 8.0) treated with RNase A (BRL; 20 micrograms/ml) and RNase T1 (BRL; 1 unit/ml) at 37°C for 45 minutes. Carbowax 8,000 (Dow Chemical Co., Midland, Mich.) and NaCl were added to 10% w/v and 0.4M respectively and the sample was incubated at 4°C overnight. The preparation was then centrifuged at 8,000 rpm for 10 minutes and the pellet resuspended in 10 mM Tris (pH 8), and 1 mM EDTA, extracted with phenol/chloroform (1:1 v/v) and precipitated with ethanol.

Physical mapping of p277-19 with restriction enzymes MspI, HinfI, ScaI, BglI, PstI, AluI and RsaI (from BRL and New England Biolabs) revealed that the plasmid had suffered a deletion from approximately nucleotide 3350 to nucleotide 3608 on the standard pBR322 map (Sutcliffe, J.G., Cold Spring Harbor Sympos. Quant. Biol., 43:77-90 (1979)). As a result of this deletion, the PstI site 3' to the insert was missing and the HinfI site at nucleotide 3362 was very close to the 3' end of the insert. The physical sequence map of the vector 5' to the insert was

unalt red. Thes findings influenc d the s l ction of the techniqu for sequencing the insert as d scribed below.

Six micrograms of the plasmid DNA were digested for 2 hours at 37°C with 24 units of MspI (New England Biolabs, Beverly, Mass.) in a 35 microliter reaction volume containing 10 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride and 1 mM DTT. The digested plasmid DNA was fractionated on a 1.2% low melting agarose (International Biotechnologies, Inc., New Haven, Conn.) gel. The largest fragment, approximately 700 base pairs in length, which was determined to contain DNA insert (via the physical mapping described above), gel by melting the agarose slice at 70°C, followed by three sequential phenol extractions, one chloroform extraction and 2 cycles of precipitation in ethanol containing 2M ammonium acetate. The DNA was resuspended in 10 microliters of water and stored at -20°C.

Approximately 1 microgram of the gel purified p277-19 DNA was digested in a 10 microliter reaction volume with six units of HinfI (BRL) at 37°C for one hour in Hin buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl) and 1 mM DTT (dithiothreitol). The reaction was stopped by heating at 65°C for 10 minutes.

The HinfI-digested DNA was then end-labelled in a 20 microliter reaction volume by adding each of dGTP, dTTP, dCTP to 50 mM and 30 x10^{-6} Ci alpha-[^{32}P]-dATP (3,000 Ci/millimole) in Hin buffer (described above) with one mM DTT and two units of the Klenow fragment of E. coli DNA Polymerase I (Boerhinger-Mannheim) at room temperature for 15 minutes. Two microliters of 0.5 mM dATP were added and the incubation continued at room temperature for 10 minutes. The reaction was stopped by heating at 65°C for 10 minutes.

Th nd-labell d DNA fragments w re fractionated in a 2% low melting temperature agarose mini g l. The fragments were electroeluted from the gel by cutting out a small w ll in front of the leading edge of th two DNA bands, filling the wells with approximately 35 microliters running buffer(0.04M Tris-acetate, 0.002 M EDTA) and continuing electrophoresis with four 45-60 second pulses (60 volts). The buffer in the wells was collected and the wells were refilled between each pulse of current.

Six micrograms of salmon sperm DNA (Sigma) were added to the DNA fragments and the mixture was extracted once with phenol and chloroform (1:1 v/v) and once with chloroform, followed by precipitation with 2M ammonium acetate and ethanol.

The precipitated DNA was resuspended in 53 microliters of water and sequenced according to the method of Maxam and Gilbert, Methods in Enzymology, Vol. 65, 499-560 (1980). The details of this method are set forth in Table I, which is based on a table of Maniatis, et al., "Recombinant DNA: A Cloning Manual" Cold Spring Harbor (1980).

The p277-19 DNA fragment encoded a peptide which contained a series of tandem amino acid repeats. The repetitive unit of the peptide was four amino acids in length and consisted of proline, asparagine, alanine and asparagine repeated 23 times in tandem. The nucleotide sequence of the DNA fragment is illustrated in Fig. 1. The sequence is aligned as a matrix with the reading frame in register with that of the beta-lactamase. The sequence was derived according to the method of Maxam and Gilbert supra using the Hpa II site 5' to the PstI insert in pBR322 and a HinfI site 3' to the insert as labelling sites. Due to the 300 base pair deletion in the pBR322 on the 3' side of the insert, the Hinf I site has been brought to within 10 base pairs of the 3' end of th d(C)-tailed cDNA insert.

TABLE I. SUMMARY OF BASE-SPECIFIC REACTIONS FOR SEQUENCING END-LABELLED DNA

	G	G & A	T & C	C	A C
					1 mg of salmon sperm DNA in each
Mix	200 µl DMS buffer 10 µl [^{32}P]DNA	10 µl H_2O 10 µl [^{32}P]DNA	10 µl H_2O 10 µl [^{32}P]DNA	15 µl 5 M NaCl 10 µl [^{32}P]DNA	100 µl { 2 N NaOH, 1 mM EDTA 5 µl [^{32}P]DNA
Chill to	0°C	0°C	0°C	0°C	Heat to 90°C 3-4 min.
Add	1 µl DMS	25 µl formic Acid	30 µl HZ	40 µl HZ	150 µl 1 N acetic acid 5 µl tRNA (1mg/ml) 750 µl 95% ethanol
Incubate	20°C, 2-3 min.	20°C, 5 min.	20°C, 8 min.	20°C, 12 min.	
Add	50 µl DMS stop 750 µl ethanol	200 µl HZ stop 750 µl ethanol	200 µl HZ stop 750 µl ethanol	200 µl HZ stop 750 µl ethanol	
Store	-70°C, 10-15 min.	-70°C, 10-15 min.	-70°C, 10-15 min.	-70°C, 10-15 min.	-70°C, 10-15 min.
Centrifuge	10 min.	10 min.	10 min.	10 min.	10 min.
To pellet add	250 µl 0.3 M NaAc 750 µl ethanol	250 µl 0.3 M NaAc 750 µl ethanol	250 µl 0.3 M NaAc 750 µl ethanol	250 µl 0.3 M NaAc 750 µl ethanol	250 µl 0.3 M NaAc 750 µl ethanol
Store	-70°C, 10-15 min.	-70°C, 10-15 min.	-70°C, 10-15 min.	-70°C, 10-15 min.	-70°C, 10-15 min.

TABLE I. CONTINUED

	G	G & A	T & C	C	A C
Centrifuge	10 min.	10 min.	10 min.	10 min.	10 min.
Rinse pellet with	70% ethanol	70% ethanol	70% ethanol	70% ethanol	70% ethanol
Vacuum dry					
To pelled add	100 µl 1.0 M piper-idine	100 µl 1.0 M piper-idine	100 µl 1.0 M piper-idine	100 µl 1.0 M piper-idine	100 µl 1.0 M piper-idine
Heat to	90°C, 30 min.	90°C, 30 min.	90°C, 30 min.	90°C, 30 min.	90°C, 30 min.
Lyophilize					
Add	20 µl H_2O	20 µl H_2O	20 µl H_2O	20 µl H_2O	20 µl H_2O
Lyophilize					
Add	10 µl H_2O	10 µl H_2O	10 µl H_2O	10 µl H_2O	10 µl H_2O
Lyophilize					
Add	10 µl loading buffer	10 µl loading buffer	10 µl loading buffer	10 µl loading buffer	10 µl loading buffer
Vortex					
Heat to					
Chill in Ice	90°C, 1 min	90°C, 1 min	90°C, 1 min	90°C, 1 min	90°C, 1 min
Load onto Gel					

Reactions should be carried out in siliconized Eppendorf tubes.

EXAMPLE VII

Presence of Repetitive Epitopes in th Immunodominant Region of CS Proteins of *P. falciparum.*

Th presence of r p titive epitopes in *P. falciparum* CS proteins was confirmed by performing a two-site immunoradiometric assay with a single monoclonal antibody. This is illustrated in Figure 2A and 2B.

In this assay wells of flexible microtiter plates (Dynatech Inc.) were coated with 20 micrograms/ml anti-*Plasmodium falciparum* monoclonal antibody (2A10). After repeated washes with phosphate buffered saline containing 1% bovine serum albumin, the wells were incubated with two fold serial dilutions of lysates of *E. coli* LE 392, containing plasmid p277-19 or *E. coli* LE 392 containing the pBR322 vector. Following a two hour incubation at room temperature, the wells were washed and 30 microliters of [^{125}I]-labeled monoclonal antibody 2A10 (1 X 10^5 cpm; specific activity 2 x 10^7 cpm/microgram) were added. After an incubation for one hour at room temperature, the wells were washed with PBS-Tween 20-BSA, dried and counted in a gamma counter. Lysates of *E. coli* LE 392 containing plasmid p277-19 were also tested using monoclonal antibody 2A10 coated plates and an unrelated [^{125}I]-labeled monoclonal antibody (X-X). As illustrated in Fig. 2A, the recombinant protein expressed by clone p277-19 simultaneously binds both the immobilized and the radiolabeled antibody. This indicates that the recombinant protein, like the authentic CS protein, contains at least two epitopes which are recognized by the anti-CS monoclonal antibody 2A10.

EXAMPLE VIII

Inhibitory Effect of Bacterial Extracts Made from LE 392 (p277-19) of the Binding of Labeled Monoclonal Antibody to the Epitopes of Authentic *P. falciparum* CS Proteins

The following inhibition assay was performed. 10 microliters (5 X 10^4 cpm) of [^{125}I]-label d monoclonal antibody 2A10 were incubated with 30 microliters of two fold serial dilutions of lysates of E. coli LE 392 containing plasmid p277-19 (O-O) or E. coli LE 392 containing the pBR322 vector (X-X). Following a thirty-minute incubation at room temperature, 30 microliters of these mixtures were transfered into microtiter plates previously coated with an extract of P. falciparum sporozoites (Zavala et al., J. Exp. Med. 157:1947 (1983)). After a one hour incubation period the wells were washed, dried and counted in a gamma counter. The results of this inhibitory assay are illustrated in Fig 2B. The results show that bacterial extracts made from LE 392 containing the plasmid p277-19 inhibit the binding of labeled monoclonal antibody to the epitopes of native P. falciparum CS proteins. The specificity of this reaction was confirmed by a further experiment in which it was shown that cell extracts of p277-19 did not inhibit the binding of an anti-Plasmodium berghei monoclonal antibody to the corresponding CS protein. These data show that the recombinant protein encoded by p277-19 exhibits the antigenic feature of the P. falciparum CS protein.

EXAMPLE IX

Amino Acid Sequence of the 4-Amino Acid Repeat

The nucleotide sequence of the p277 19 insert was derived according to the method of Maxam and Gilbert, Proc. Nat'l. Acad.(USA) 74:560 (1977) using the Hpa II site 5' to the PstI site insert in pBR322 and a HinfI site 3' to the insert as labelling sites. The nucleotide sequence of the p277-19 insert is illustrated in Fig. 1. The method followed is described in detail in Table I.

Th deduced amino acid sequence of the f ur amino acid repeat set forth b low is bas d upon translation of the nucl otide sequence in th correct r ading frame: Pro-Asn-Ala-Asn. (All sequences are express d from th end nearest the NH_2 terminus on the left to the end nearest the -COOH terminus on the right.)

The four amino acid sequence is repeated twenty-three times in tandem. However, at the nucleotide level, the repeats in p277-19 consist of eight variants. Both of the asparagine codons, three of the four proline codons, and two of the four alanine codons are used (Fig. 1). This repetitive pattern of four amino acids is shorter than any of the three known CS protein repeats. The repeats of P. knowlesi and P. cynomolgi (Gombak strain), two simian malaria parasites, are twelve and eleven amino acids long, respectively (Godson, et al. Nature 305:29 (1983); V. Enea et al. PNAS submitted (1984).

Although neither the DNA nor the protein sequences of these three sets of repeats exhibit extensive homology, they have similarities in their amino acid composition. Alanine and asparagine are present in the repeats of all three CS proteins; proline is present in P. knowlesi and P. falciparum; and glutamic acid and glycine are present in P. knowlesi and P. cynomolgi (Gombak).

The CS protein of P. falciparum appears to be encoded by a single copy gene based on the results of genomic DNA mapping experiments. In outline, the genomic clone was mapped as follows:

P. falciparum DNA obtained from blot stages was digested with restriction enzymes (including EcorI, BamhI, HindIII, BglII, SalI, XhoI) fractionated on agarose gel, transferred to a nitrocellulose filter, hybridized with [^{32}P]-labeled p277-19 and autoradiographed. This procedure permits the determination of the sizes of the P. falciparum DNA (generated by all the above restriction

fragments] that bears homology to th radi active prob . Sp cifically, the SalI digest generated a fragment f approximat ly 7,000 nucleotides that hybridized with the probe. Since a fragment of this size was significantly smaller than the bulk of the fragments generated by SalI, a size-fractionation of SalI-digested DNA was undertaken to obtain the 7,000 nucleotide fragment generated by SalI which was expected to constitute a significant enrichment for the CS-gene.

SalI-digested DNA was fractionated on a sucrose gradient (10-40% w/v in 1M NaCl, 2mM Tris-HCl (pH 8) and 5mM EDTA; SW-41) at 38,000 rpm at 20°C for 16.5 hours. The fractions were collected and aliquots were hybridized to [^{32}P]-labelled p277-19.

The fraction that contained the CS sequence was ligated to SalI-digested EMBL4-DNA. (EMBL4 is a derivative of phage lambda; other SalI-digested phage lambda DNA vectors could have been employed, such as Charon 28 obtainable from BRL.)

The ligate was packaged in vitro (packaging extracts and protocols are commercially available from BRL and other sources) and plated on LE 392. The resulting plaques were screened with [^{32}P]-labelled p277-19. Two independent positive plaques were thus identified.

Characterization of the isolates is conducted by well-known techniques and includes physical mapping of the phages, subcloning of specific DNA fragments into plasmid vectors, determination of the DNA sequence of these fragments and, if necessary, mapping experiments with the messenger RNA of the P. falciparum CS protein. Using this procedure, the gene coding for the entire CS-protein of P. falciparum is isolated and sequenced.

EXAMPLE X

Synth sis of Peptid s Having the R peating Amino Acid S quence

To confirm that the pr c ding amino acid s quence contains the immunoreactive site, a corresponding synthetic peptide has been synthesized using solid phase resin synthesis (Marglin, H. and Merrifield, R. B., Ann. Rev. Bio. Chem. 39:841-866 (1970). The general steps of the peptide synthesis techniques used herein are well known. The synthesis was carried out using a benzhydrylamine (BHA) resin on an automated synthesizer controlled by a computer using a program based on that of Merrifield, R. B., Fed. Proc. 21:412 (1962); J. Chem. Soc. 85:2149, (1963). The four amino acid repeat was assembled on the benzhydrylamine resin. The tandem repeat was assembled by the sequential addition of protected amino acids in the same order as the four amino acid repeat, using the method described above. Amino acid composition and sequence analysis performed by automated Edman degradation confirm that the peptide had been correctly synthesized. A 12-MER peptide was thus synthesized which consisted of three sequential repeats of the minimum repeating unit (Pro-Asn-Ala-Asn).

To confirm that the correct epitope has been obtained, rabbits are immunized with a peptide consisting of three and six tandem repeats of the four amino acids coupled to a carrier (bovine gamma globulin in complete Freund's adjuvant). Four weeks after the injection, the rabbits are bled and their serum assayed for the presence of antibodies against the tandemly repeated peptides and against extracts of _P. falciparum_ sporozoites. The results show that the animals produce high titers (greater than 1:1000) of antibodies to the native CS protein present in the parasite extracts.

EXAMPLE XI

Inhibition of the Binding of Monoclonal Antibody To Authentic P. falciparum Antigen by the Synth tic P ptide

The antigenicity of a synthetic 12-amino acid peptide consisting of a 3X tandem repeat of the minimum repeating unit (Pro-Asn-Ala-Asn) of the *P. falciparum* CS protein was confirmed by a direct radioimmunoassay, as follows:

P. falciparum sporozoite extract was used to coat the bottom of microtiter well plates (as previously described). Unbound native antigen was removed by washing and the wells were filled with serial dilutions of PBS-BSA containing serial dilutions of the synthetic 12-amino acid peptide having the sequence (Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn.) Control wells were filled with serial dilutions of PBS-BSA containing the synthetic 12-amino acid peptide representing the epitope of *P. knowlesi*, i.e. (Gln-Ala-Gln-Gly-Asp-Gly-Ala-Asn-Ala-Gly-Gln-Pro). Saturation amounts of [^{125}I]-labelled monoclonal antibody 2A10 were then added to the wells (8 x 10^4 cpm) and allowed to bind. After removal of the supernatant residual radioactivity was measured with a gamma counter. The results are shown in Table II.

TABLE II

Well No.	1	2	3	4	5	6
(1) *P. falciparum* 12-peptide (micrograms/ml)	500	50	5	0.5	0.05	0.005
(2) Residual Radio-activity of (1) (cpm)	194	297	1590	4990	6092	6271
(3) Non-Specific Antigen (*P. knowlesi* 12-peptide) (micrograms/ml)	500	50	5	0.5	0.05	0.005
(4) Residual Radio-activity of (3) (cpm)	5179	5838	6170	6409	6174	6181

Control on wells coated with BSA alone without sporozoite extract showed a residual radioactivity of 27-58 cpm.

The above results show that the monoclonal antibody recognizes and quantitatively binds to the synthetic 12-amino acid peptide.

EXAMPLE XII

Recognition of the Synth tic P ptide By Monoclonal Antib dies to P. falciparum CS-Protein

Another immunoradiometric assay was used to show that the synthetic 12-amino acid p ptide is r cognized by several antibodies to P. falciparum CS protein. The antibodies used are designated 2A10, 1E9, 3D6, and 2C11.

A synthetic 12-MER peptide (three repeats of the pro-asn-ala-asn peptide) (20 micrograms/ml) was bound to the bottom of microtiter wells as previously described. The wells were saturated with BSA.

Serial dilutions of each type of unlabelled monoclonal antibody preparation (10 micrograms/ml) in serial dilution were introduced into separate 12-MER coated wells, and sufficient time was allowed for the antibody to bind to the coat.

Finally, after washing the wells, saturation amounts of affinity-purified, radiolabeled goat anti-mouse IgG were also introduced into the wells and allowed to bind to the monoclonal antibodies bound to the peptide coat. The wells were then washed and residual radioactivity was measured in a gamma counter. The results are summarized in Table III below.

Unlabelled monoclonal antibodies to P. knowlesi, BSA coated wells (in the absence of anti-P. falciparum monoclonal antibody) and 12-amino acid peptide coated wells (in the absence of anti-P. falciparum monoclonal antibody) were used as controls. Controls showed 40-100 cpm.

TABLE III

Well No.	1	2	3	4	5
Unlabelled Antibody (micrograms/ml)	10	0.1	0.01	0.001	1×10^{-4}
	Residual Radioactivity (cpm)				
2A10	1844	775	96	90	71
1E9	3787	2475	888	176	86
3D6	457	119	121	83	78
2C11	2874	1761	863	296	107

The controls in which the wells were incubated using dilutions of three other non-specific monoclonal antibodies of the same isotype resulted in residual radioactivity ranging between 44 and 100.

The above results show that several monoclonal anti-P. falciparum antibodies recognize and bind quantitatively to the synthetic 12-amino acid peptide.

EXAMPLE VIII

Immunization with the Synthetic R peat d Epitope of P. falciparum (12 MER and 24 MER)

A tandemly repeated peptid (3X and 6X) is synthesized as described above, except a cysteine residue is added at the N-terminus. To determine whether the synthesis had been performed correctly, an aliquot is subjected to acid hydrolysis at reduced pressure (5.6M HCl 110°C, 72 hours) and its amino acid composition is determined. The peptide is coupled to a carrier protein (e.g. keyhole limpet hemocyanin, or tetanus toxoid, through its N terminal cysteine residue, using a m-malemidolbenzoyl-N-hydroxysuccinimide ester (MBS) as the coupling reagent (Ling et al, Biochemistry 18, 690 (1979)). This is a bifunctional reagent which under appropriate conditions reacts with the amino group of the carrier and with the third group of the peptides. 4 mg of the carrier protein in 0.25 ml of 0.05 PO_4 buffer, pH 7.2, is reacted dropwise with 0.7 mg MBS dissolved in dimethyl formamide and stirred for 30 minutes at room temperature. The product MB carrier is separated from the unreacted chemicals by passage in a Sephadex C-25 column equilibrated in 0.05 M PO_4 buffer, pH 6.0. The MB carrier is then reacted with 5 mg of the 12- or 24-MER containing compound, dissolved in PBS (pH 7.4.) The mixture is stirred for 3 hours at room temperature and coupling is monitored with radioactive peptide. The conjugate is dialyzed and used as a vaccine for administration to non-human primates in a physiologically acceptable medium.

Alternatively, the tandemly repeated peptide (3X) can be further polymerized with glutaraldehyde as follows: Dissolve 20 mg of peptide in 10 ml of phosphate buffered saline (PBS). Make fresh glutaraldehyde from a stock with 13 milliliters of PBS. Stir the peptide and glutaraldehyde overnight at room temperature. Neutralize the excess glutaraldehyde with 1M ethanolamine. Separate the polymerized p ptide by high performanc

liquid chromatography (HPLC) using sizing columns, and dialyz repeatedly against wat r. This is then us d in a vaccine preparation.

Fiv chimpanzees are immunized with 200 micrograms of the conjugated protein or the polymerized product absorbed to aluminum hydroxide gel. Their serum is monitored for the presence of antibodies to CS proteins of P. falciparum using an immunoradiometric assay. Serum dilutions are incubated in antigen-coated wells of microtiter plates. The presence of chimpanzee antibody bound to the solid-phase antigen is monitored by incubation with [^{125}I]-labeled affinity-purified rabbit-anti-human IgG (which strongly cross-reacts with chimpanzee IgG).

After 30 days, the serum titer of the chimpanzees rises to titers of greater than 1/1000. At this time, these chimpanzees (as well as five other control chimpanzees injected with non-conjugated carrier protein adsorbed to aluminum hydroxide) are challenged with 2,000 viable P. falciparum sporozoites. The infection is monitored daily for a total of 30 days by microscopic examination of blood smears, starting one week after the inoculation of the parasites. The results show that the five chimpanzees immunized with the vaccine (conjugated protein) are totally protected, that is, no parasites are found in their blood. In contrast, the control chimpanzees have trophozoites of P. falciparum in their circulation 10-12 days after challenge. Based on the close similarities of human and chimpanzee immune responses and on the fact that protection immunity has been obtained in humans by injection of inactivated sporozoites of P. falciparum, the results obtained upon immunization of chimpanzees with the described synthetic peptide will also be obtained following similar treatment of human patients.

What is claimed is:

1. A peptide comprising the amino acid sequence (pro-asn-ala-asn).

2. A peptide comprising the peptide of claim 1 tandemly repeated at least 23 times.

3. A vaccine against malaria comprising as an active ingredient the peptide of claim 1 and a carrier.

4. A vaccine against malaria according to claim 3 wherein said peptide is adsorbed or covalently attached to a carrier protein.

5. The peptide of claim 1 wherein said amino acid sequence corresponds to an epitope of the CS protein of a sporozoite of the species Plasmodium falciparum.

6. The peptide of claim 4, wherein the amino acid sequence corresponding to an epitope of a CS protein of plasmodium falciparum is chemically synthesized.

7. A agent which neutralizes the infectivity of Plasmodium falciparum sporozoites comprising a peptide according to claim 1 coupled to a carrier.

8. A synthetic antigen comprising the amino acid sequence (pro-asn-ala-asn).

9. A synthetic antigen comprising the amino acid sequence of claim 8 tandemly repeated at least twenty-three times without variation.

10. A vaccine against P. falciparum sporozoites comprising the synthetic antigen according to claims 7 or 8 in a physiologically acceptable medium.

11. A vaccin for immunizing a mammal against malaria comprising th synthetic antig n according to claim 1 adsorbed or covalently attach d to a carrier protein, in a physiologically acceptable m dium.

12. A vaccine according to claim 11 wherein said synthetic antigen is immunochemically reactive with a monoclonal or polyclonal antibody to a sporozoite CS protein of the species Plasmodium falciparum.

13. A DNA fragment comprising a deoxynucleotide sequence coding for the peptide of claim 1.

14. A DNA fragment consisting essentially of a deoxynucleotide sequence coding for the amino acid sequence (pro-asn-ala-asn) tandemly repeated twenty-three times.

15. A recombinant DNA molecule comprising an inserted DNA fragment consisting essentially of a deoxynucleotide sequence coding for the amino acid sequence pro-asn-ala-asn.

16. The DNA fragment of claim 14 wherein said DNA fragment is inserted at a site suitable for expression of the coding sequence, either directly or as a fusion protein.

17. A microorganism transformed by an expression vector comprising an inserted DNA fragment according to claim 14.

18. The microorganism of claim 16 comprising E. coli.

19. A synthetic peptide comprising the amino acid sequence (pro-asn-ala-asn),

20. The synthetic p ptide of claim 19, wherein said sequence is tandemly repeat d up to twenty-three tim s.

21. A tand mly repeating p ptid comprising an epitope of the CS protein of the species Plasmodium falciparum.

22. A peptide comprising an epitope of a sporozoite CS protein of a member of the species Plasmodium falciparum and having a tandemly repeating sequence of four amino acids, said tandem repeat having a combined molecular weight of less than 3,000.

23. A synthetic peptide comprising (pro-asn-ala-asn-pro-asn-ala-asn-pro-asn-ala-asn).

24. A method for raising antibodies to CS antigen of P. falciparum sporozoites which comprises administering to a host an effective amount for raising antibodies to CS antigen of a protective peptide comprising (pro-asn-ala-asn).

25. The method of claim 24 wherein said peptide is tandemly repeated at least twenty-three times.

26. A peptide according to claim 1 tandemly repeated three times.

27. A peptide according to claim 1 tandemly repeated six times.

28. A peptide having an amino acid sequence consisting essentially of a subsequence of four amino acids, said subsequence defining an immunodominant epitope of a repeating unit of a tandem repetitive polypeptide of P. falciparum protein, said repeating unit being longer in length that said peptide.

	Pro	Asn	Ala	Asn	
G^{15}	CCA	AAT	GCA	AAC	
	C	T	A	C	
	A	C	A	C	
	C	T	A	T	
	T	T	A	C	
	C	T	A	T	
	T	T	A	T	
	T	T	C	T	
	A	T	A	T	
	T	T	A	C	
	C	T	A	T	
	T	T	A	T	
	T	T	C	T	
	A	T	A	T	
	A	T	A	C	
	A	C	A	C	
	C	T	A	T	
	T	T	C	T	
	A	T	A	T	
	A	T	A	C	
	A	T	A	C	
	A	T	A	C	
	C	T	A	T	
	CCT	AAT	AAA	AAC	
	AAT	CAA	GCC	CCC	C^{18}

FIG. 1

FIG. 2A

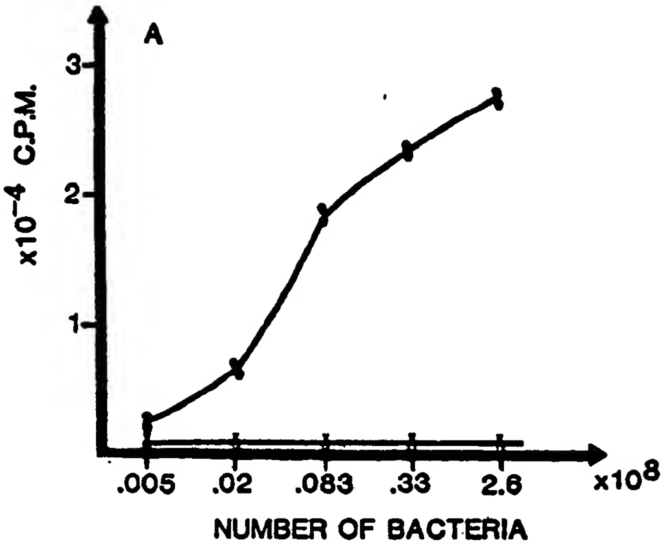
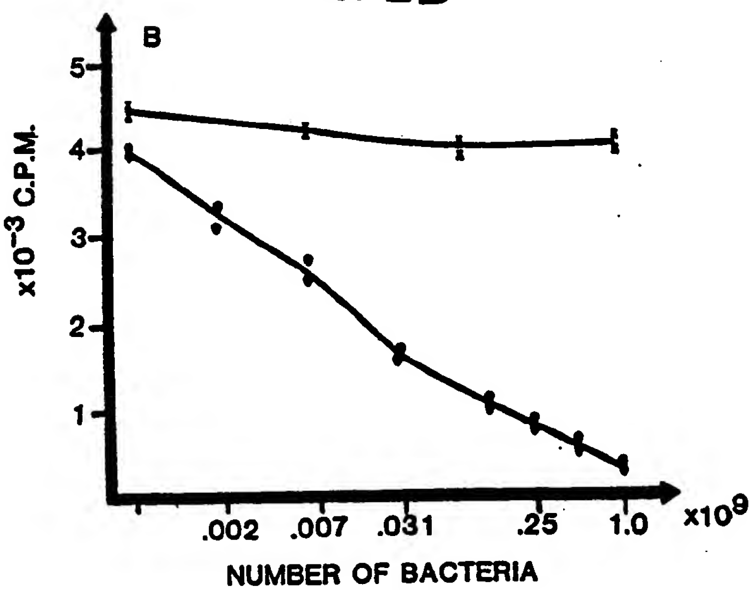

A
3
2
1
x10⁻⁴ C.P.M.
.005
.02
.083
.33
2.6
x10⁸
NUMBER OF BACTERIA

FIG. 2B


B
5
4
3
2
1
x10⁻³ C.P.M.
.002
.007
.031
.25
1.0
x10⁹
NUMBER OF BACTERIA

INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/01416

I. CLASSIFICATI N F SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. CL. -4-C07K 7/02;C07K 7/06;A61K 39/00;A61K 39/12;C12P 21/02;
C12P 19/34; US. CL. 260/112.5R;424/88;424/89;435/70;435/91 -3-

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	260/112.5R; 424/88;424/89; 435/70;435/91

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	N, J. Exp. Med., Vol. 157, pages 1947-57, Issued June 1983, Zavala, et al.	1-6,8-11 & 19-28
X	N, Biochemistry, Vol. 23, pages 5665-70, Issued 1984, Schlesinger, et al.	1-6,8-11 & 19-28
X	N, Science, Vol. 220, pages 1285-88, Issued June 1983, Lupski, et al.	1-28
L&X	N, Chemical Abstract, Vol. 102, page 40904d, Vincenzo, et al.	1-28
X	US, A, 4,466,917 Published 21 Aug.1984 Nussenzweig, et al.	1-28

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
01 Oct. 1985	18 OCT 1985
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Delbert R. Phillips

Form PCT/ISA/210 (second sheet) (October 1981)

FURTHER INFORMATI N C NTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ______, because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ______, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1,2,19-23 and 26-28
II. Claims 3-12,24 and 25
III. Claims 13-18,29 and 30

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☒ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.